

DETECTION OF *COXIELLA BURNETII* (Q FEVER) AND *BORRELIA BURGDORFERI* (LYME DISEASE) IN FIELD-COLLECTED TICKS FROM THE CAYO DISTRICT OF BELIZE, CENTRAL AMERICA

by

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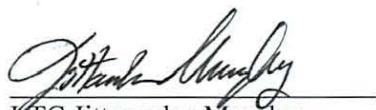
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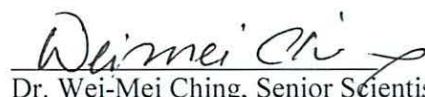
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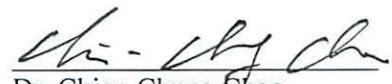
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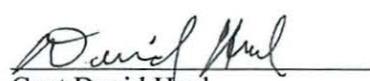
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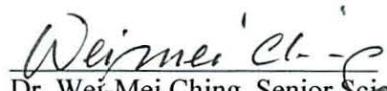
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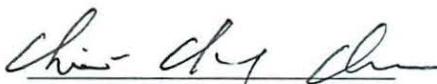
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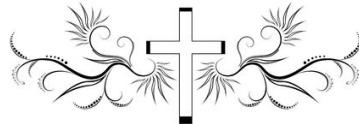
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DEDICATION

“I can do all things through Christ who strengthens me.”

-Philippians 4:13

Thank you, God, for reminding me of that scripture everyday during my graduate studies. It is only by your hand and your hard work that I am able to submit this thesis with pride.



To my husband, Jonathon, thank you for having faith in me. You uprooted your life to support me and be by my side through this “boring thesis.” Over these last two years, we’ve begun an amazing new chapter in our lives. Leaving with my new degree in hand and our new baby boy, Abram, brings me great joy for our many blessings. You will always be my hero.

Lastly, during my research, the entomology community lost a very dear mentor, Dr. Jimmy K. Olson. Dr. Olson, I deeply miss not having our regular inspiring phone chats. While writing this thesis, my recommendation letter from you is pinned to my wall; reminding me of the constant encouragement you had for me to obtain my masters degree. You always believed in me, even when I lost hope in myself. Your words continue to uplift me, whether they are from your old typewriter or the soft whispers on my heart. We’ll see each other soon in His divine kingdom.

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Amanda A. Cline
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ABSTRACT

Detection of *Coxiella burnetii* (Q fever) and *Borrelia burgdorferi* (Lyme disease) in field-collected ticks from the Cayo District of Belize, Central America

Amanda A. Cline, Masters of Science in Public Health (Medical Zoology), 2016

Thesis directed by: CAPT David Hoel, Ph.D., Assistant Professor, Division of Tropical Public Health, Department of Preventive Medicine and Biostatistics (PMB), USUHS, Bethesda, Maryland

Background: Little documented tick-borne disease data exists for Central America. Several tick-borne diseases are suspected of occurring in Central America, based on patient symptoms, and a need to test local tick populations for various tick-borne diseases and to describe their possible presence and geographical distribution may assist public health authorities in prevention and control efforts. For this project, real-time quantitative PCR (qPCR) methods are used to screen for *Coxiella burnetii* (Q fever) and the bacterial spirochete *Borrelia burgdorferi* (Lyme disease) in the tick population of the Cayo District of central Belize.

Methods: A total of 272 field-collected ticks (97 tick pools) were previously collected in Belize from tick drags and host collection methods for this study. The wet season (November) yielded 180 specimens and the dry season (February) yielded 92 specimens. Ticks were cut in half, DNA extracted and screened for the above-mentioned pathogens using qPCR methods. qPCRs were conducted for each pathogen and repeated reactions for positive confirmation performed on an ABI 7500 FAST real time PCR

instrument and program to investigate their prevalence in tick pools from Belize. Fisher's exact test was used to determine the association between climatic conditions and presence of *B. burgdorferi* and *C. burnetii*.

Results: All 97 tick pools collected were tested for the presence of *C. burnetii* (Q fever) and *B. burgdorferi* (Lyme disease) using SYBR Green-based qPCR assays as described previously. Of these, 0 (0%) ticks tested positive for the presence of *C. burnetii* DNA, of which 1 possible positive specimen could be identified. Three (3%) tick pools tested positive for the presence of *B. burgdorferi* DNA, one positive pool containing five male *Amblyomma maculatum* ticks and two tick pools containing 20 each of *Dermacentor nitens* larvae. *C. burnetii* was not positively identified in this study, but prevalence for *B. burgdorferi* was analyzed. No co-infections occurred among the 97 ticks pools sampled.

Conclusions: This study is the first to document tick-borne *B. burgdorferi* in Belize and also the first to test for tick-borne *C. burnetii* in this region of Central America. PCR analysis determined the presence of *B. burgdorferi* and revealed the need for modified research in regard to aiming for a different target gene when testing for *C. burnetii*. No occurrence of *B. burgdorferi* and *C. burnetii* co-infections were found in this study. Fisher's exact test determined no significant association between pathogen presence and climatic conditions. Future use of molecular techniques (DNA barcoding), to verify vector genus and species in positive results is ideal before scientific publication of results from this study. With focus on *B. burgdorferi*, the primary infected species was *Dermacentor nitens*.

Key Words: Belize, Lyme, Q fever, *Coxiella burnetii*, *Borrelia burgdorferi*, co-infection, Latin America, Central America, Tick-borne disease, *Dermacentor nitens*, *Amblyomma mixtum*

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CHAPTER 1: Introduction

Lyme disease/Lyme borreliosis and Q Fever can both be vectored by bites from infected ticks. Both agents are pathogens of vertebrate hosts, and ticks become infected while taking a blood meal. Many species of ticks parasitize birds, while other tick species parasitize deer or other migratory animals. Recently, the importation of reptiles has become a concern on the dissemination of disease from tick populations [8]. One study in North America found there are at least 20 species of birds that are competent reservoirs for *Borrelia burgdorferi* and some of those birds are migrating species [24]. When researching how animal migrations are affecting human health, ticks become a secondary factor. Tick-borne diseases in Central America are poorly documented and this study may assist in filling gaps for known pathogens in the region.

The National Institutes of Health (NIH) lists Lyme borreliosis in the top emerging pathogens or diseases and the Centers for Disease Control and Prevention (CDC) places Lyme disease on their priority list of emerging and reemerging pathogens or diseases [34]. Although eliminating Lyme disease spirochetes from nature is unrealistic, diminishing their threat to humans would seem to be an achievable goal [45]. Lyme disease has frequent co-infections with many other pathogens of concern [3]. Infected ticks acquire *B. burgdorferi* from their host while feeding, but more frequently have shown transstadial infection [13].

Worldwide, *C. burnetii*, the agent of Q-fever, infects a wide variety of ticks, domestic livestock, and other wild and domestic mammals and birds. Although tick-borne transmission of Q fever to humans is uncommon, a documented case of crushing an infected tick between the fingers resulted in a Q fever infection [14]. Most human

infections originate from contact with heavily infected birth products of sheep, goats, and cattle as it occurs on farms, in research laboratories, and in slaughterhouses. *C. burnetii* is also shed in milk, urine, and feces of infected animals [55]. Walker further writes that animals are most likely to become infected by aerosol means and by the bite of any of the 40 species of ticks (as of 1996) capable of carrying this pathogen. Hard and soft ticks may become infected during feeding and may also transmit *C. burnetii* transovarially and transstadially and excrete it via feces, saliva and coxal fluid [1,43,49,57]. In humans, presentation of Q fever ranges from asymptomatic, to acute disease, to chronic illness. In the majority of cases, acute disease presents as a self-limiting febrile illness with half of cases suffering severe headaches. In severe cases of acute disease, atypical pneumonia is often found [51].

This study is the first to investigate the presence of *B. burgdorferi* and *C. burnetii*, in the tick population in Belize and attempted to determine the plausibility of co-infections between these pathogens among the tick population in central Belize.

TICKS OF BELIZE

According to conversations with several entomologists who have worked in different areas of Central America, several disease-carrying tick species occur and have been collected in Belize, however, none have been documented. Since no published material exists illustrating the geographical distribution of tick species in Belize, identifying gender, life stage, and species was carefully conducted using a dichotomous key. Keys by Guzman-Cornejo et al. [22] were used to identify most ticks species, however, keys by Keirans & Litwak [30] were used for the genus *Amblyomma*, with verification from a subject matter expert. Ticks assumed to live in Belize might also be

deduced from published articles listing tick species in Mexico and Panama [4,16]. Table 1 (below) lists the genus and species of hard ticks identified and collected from the Cayo District of Belize used in this study.

Table 1: Species of Belize ticks used for study

<i>Amblyomma mixtum</i>
<i>Amblyomma maculatum</i>
<i>Amblyomma oblongoguttatum</i>
<i>Amblyomma ovale</i>
<i>Dermacentor nitens</i>
<i>Ixodes affinis</i>
<i>Rhipicephalus microplus</i>
<i>Rhipicephalus sanguineus</i>

Amblyomma mixtum

Previously classified as *Amblyomma cajennense*, *Amblyomma mixtum* emerged as its own species based on morphological differences and is a common tick occurring in Central America [38]. This tick has been found as far north as Texas and extends south to Ecuador, including Panama and the Caribbean islands [2]. Because of the recent name change, research can be difficult in determining vector competency and capabilities. Questing behavior has been documented [12], but other bionomics information is limited. However, *C. burnetii* specific DNA was identified from two *A. mixtum* tick pools collected from a domestic horse in Cuba in a recently published article [39].

Amblyomma maculatum

Amblyomma maculatum, the Gulf Coast tick, is a significant pest of livestock, predominantly cattle, from South America to the southern USA. *A. maculatum* has been found infected with rickettsial organisms of unknown pathogenicity for humans in Peru [5] in areas of the country where ideal tick habitats are found. These habitats include

warm areas with prominent rainfall. This tick has also shown to be the primary vector of *Rickettsia parkeri* (the “maculatum agent”), and in some tick populations as many as 50% of individual ticks may be infected with this pathogen [41]. Feeding on a range of wild and domestic animals, the immature stages (larvae and nymphs) are commonly seen parasitizing birds. Two *A. maculatum* ticks were found infected with *B. burgdorferi* in an Arkansas study in 2012 [18].

Amblyomma oblongoguttatum

Amblyomma oblongoguttatum has been found on various hosts in South and Central American countries, including Belize. Although this tick is not strictly associated with any host, it is mostly found on birds and mammals (mainly dogs, but occasionally humans) [21].

Amblyomma ovale

Feeding mainly on carnivores, *Amblyomma ovale* has been found in Central and South America from Argentina to southern Mexico [21]. Not fixated on wild carnivores, *A. ovale* can be found on domestic animals, like dogs in Brazil [17] and have been found on humans [7].

Dermacentor nitens

Dermacentor nitens is a one-host tropical horse tick and considered an important vector for veterinarians. Originally parasitizing deer in the forests of northern South America, this tick shifted focus and adapted to horses and other livestock. Most of *D. nitens*’ life is spent in the ears of their hosts, but can also be found around the nose,

ventral abdomen, or even the perianal area. *D. nitens* transmits *Babesia caballi* transovarially (all life stages are considered important vectors) and is of importance to the horseracing industry [50]. It also is an experimental vector of *Anaplasma marginale* to cattle [27]. The first record of *B. burgdorferi* in *D. nitens* was isolated from Brazil in 2008 from ticks collected from horses [19].

Ixodes affinis

Ixodes affinis commonly infests birds and now ranges from Virginia to Argentina. Although *I. affinis* and *I. minor* rarely bite humans, it appears to be more important than *I. scapularis* as enzootic vectors of *B. burgdorferi* s.l. Presumably, they play a role similar to that of *I. spinipalpis* in the Western U.S. [6], but their role in Belize is unknown.

Rhipicephalus microplus

Previously classified under the genus *Boophilus*, *Rhipicephalus microplus* is more commonly known as the cattle tick. As its name suggests, the cattle tick primarily feeds on cattle and other livestock (goats, pig, sheep, and horses). *R. microplus* is considered the world's most important tick parasite of livestock and has been introduced from the bovid- and cervid-inhabited forests of the Indian region to many areas of tropical and subtropical Asia, northeastern Australia, Madagascar, the coastal lowlands of southeastern Africa to the equator, and much of South and Central America, Mexico, and the Caribbean [27]. Although still occasionally found in the US along the Mexico border, this tick's US population has greatly declined after a very long and expensive eradication program from 1906-1943 to eliminate "cattle-fever" (Babesiosis) from the states.

Rhipicephalus sanguineus

The “brown dog tick” or “kennel tick,” *Rhipicephalus sanguineus*, can be found on dogs worldwide. This tick obtained its name for this host preference and also its affinity for the indoors. Other than dogs, this tick can be found on wild and domestic carnivores, sheep, goats, camels, other livestock, and various wild mammals. People are seldom attacked, more often in circumstances of children playing and sleeping in close contact with heavily infested dogs [27]. Some of the many disease agents found in *R. sanguineus* include *Babesia canis*, Crimean-Congo hemorrhagic fever virus, *Ehrlichia canis*, *Rickettsia conorii*, *Rickettsia massiliae*, *Rickettsia rickettsii*, *Rickettsia rhipicephali*, and Thogoto virus. *R. sanguineus* has not been shown to transmit Lyme disease-causing spirochetes [33].

SEASONS AND GEOGRAPHY OF BELIZE

Critical influences for oviposition and the development of tick larvae are temperature and humidity [28]. There are two seasons in Belize, the dry and wet seasons. The dry season extends from January to May and the wet season covers from June to December [42]. By using collected ticks from each season (wet and dry), one can understand how climatic conditions influence risk. With infestation of migratory birds, international trade of livestock and other animals, and favorable tropical climatic conditions promoting their life cycle, the geographical range of ticks and their pathogens are expanding [26].

Belize is geographically situated in Central America, with Mexico to the north, Guatemala to the west and south, and the Caribbean Sea to the east (Fig. 1). Honduras does not have a direct land border, but is connected by sea. The country is divided administratively in six districts, from north to south: Corozal, the slowest growing population (1.5% per year); Orange Walk; Cayo which includes the capital Belmopan



Figure 1: Districts of Belize (MapCruzin)

and the universities; Belize with Belize City as the largest city in the country and a commercial center and port, and Stann Creek and Toledo, both in the southern part of the country [29]. Primarily an agricultural District, Cayo contains the majority of the livestock that ticks prefer, making it the ideal study site for Belize. Expanding globalization, increases in trade and international travel (tourism, business) coupled with deforestation (habitat modification) and bird migration have expanded the range of

disease agents to areas previously non-endemic [48].

PROJECT OBJECTIVES

The overall objective of this project is to detect the presence of the pathogens *C. burnetii* (Q fever) and *B. burgdorferi* (Lyme disease), in field-collected ticks from the Cayo District of Belize.

Specific Aims

- Determine the presence of tick-borne *Borrelia burgdorferi* and/or *Coxiella burnetii* in the Cayo District of Belize, Central America
- Determine occurrence of tick-borne *Borrelia burgdorferi* and *Coxiella burnetii* co-infections in the Belize tick population.
- Conclude whether a significant association between pathogen presence and climatic conditions exist.
- Verify vector genus and species with molecular assays in positive results (as time permits/if any).

There is a lack of documentation of vector-borne disease occurrence in Central America in general and in Belize in particular. There are several published human studies investigating the spread of rickettsioses similar to Q fever and other tick-borne pathogens such as Lyme disease in the United States and Europe, with the nearest study to Belize in Honduras [10] and Cuba [39], however, the Honduran case was not confirmed. Figure 2 illustrates the southern movement of human cases of *Rickettsia rickettsii* in Mexico from 1940-2004, indicating how these pathogens can spread. Recent reviews of Belize vector-borne pathogens associated with human disease only indicate a malaria risk to the

population and how environmental factors affect local mosquito population [23]. Central American research into the presence of vector-borne diseases include studies in dogs in Nicaragua [56], dogs in Costa Rica [53], cats in Honduras [36], birds in Costa Rica [40], dogs and goats in Brazil [35], and in many deer-related studies. However, at this time, Belize has only one published rickettsial records from central and southern Belize [31,32]. It is anticipated parts or all of this study will be published in a peer-reviewed scientific publication.



The map shows the outline of Mexico and its states. Several regions are labeled with the year they were first detected: '1940s' is labeled in the northern states of Sonora, Chihuahua, Coahuila, Nuevo Leon, Tamaulipas, and the eastern coastal states; '2004' is labeled in the southeastern state of Quintana Roo.

Figure 2: Map of Mexico*

*showing the period and regions where human cases caused by *Rickettsia rickettsii* were detected. [58]

CHAPTER 2: Materials and Methods

PRELIMINARY STUDIES

This study is a follow-on study to USUHS PhD candidate, Suppaluck Polsomboon’s “Detection of Rickettsial pathogens in ticks from Belize, Central America” dissertation. Her yet unpublished work investigates the spotted fever group (SFG) and other rickettsial pathogens from field-collected ticks from several districts in Belize. Polsomboon’s work alludes to the possibility of other rickettsial pathogens in the same area, as do the previously mentioned references [35,36,40,53,56]. Polsomboon has found positive SFG tick pools in her study and the possibility of other rickettsial and tick-borne pathogens migrating into Belize is now realized. The same tick DNA was shared for this study with permission of the Naval Medical Research Center (NMRC).

COLLECTION METHOD AND IDENTIFICATION

As previously stated, tick specimens were shared from the Naval Medical Research Center (NMRC) with permission from the Viral and Rickettsial Diseases Department. A total of 272 ticks, including 184 adults, 35 nymphs, and 53 larvae, were collected from animals and tick drags in the Cayo District of Belize during November (wet season) 2014 and February (dry season) 2015; host association and species identification of each specimen are provided in Table 2. The Belize Ministry of Health provided host-collected ticks. Ticks were placed in labeled vials of 70% ethyl alcohol and variables (soil moisture, vegetation types, and GPS coordinates) were recorded at collection sites. The wet season (November) yielded 180 samples and the dry season (February) yielded 92 samples. Polsomboon, with assistance from Dr. Richard Robbins

of the Armed Forces Pest Management Board, identified ticks to species using the referenced dichotomous keys.

Table 2- Tick Samples Collected in the Cayo District of Belize								
Month	Location/ tick species	No. of ticks					Hosts/Drag	# of pools
		male	female	nymph	larva	Total		
November (wet season)	<i>A. mixtum</i>	21	11	1	0	33	Horse/Dog	13
	<i>A. maculatum</i>	13	4	0	0	17	Dog	12
	<i>A. ovale</i>	0	1	0	0	1	Dog	1
	<i>D. nitens</i>	7	15	10	0	32	Horse	7
	<i>R. sanguineus</i>	1	3	0	0	4	Dog	4
	<i>R. microplus</i>	1	3	1	0	5	Cow	3
	Total Host Ticks	43	37	12	0	92		40
	Total Tick Drag					0		
November Total		43	37	12	0	92		40
February (dry season)	<i>A. mixtum</i>	26	11	15	0	52	Dog/Horse	18
	<i>A. maculatum</i>	24	6	2	0	32	Dog	15
	<i>A. oblongoguttatum</i>	1	2	0	0	3	Dog	2
	<i>A. ovale</i>	1	3	0	0	4	Dog	4
	<i>D. nitens</i>	9	18	4	0	31	Horse	9
	<i>I. affinis</i>	0	2	0	0	2	Dog	2
	<i>R. sanguineus</i>	0	1	1	0	2	Dog	2
	Total Host Ticks	61	43	22	0	126		52
	<i>Dermacentor nitens</i>	0	0	0	52	52	Drag	3
	<i>Amblyomma mixtum</i>	0	0	1	1	2	Drag	2
Total Tick Drag						54		5
February Total		61	43	23	53	180		57
Total		104	80	35	53	272		97

TICK PROCESSING AND DNA EXTRACTION

Adult ticks were cut in half, with half used for DNA extraction and the other half preserved at -80°C for further testing if necessary and possible DNA barcoding. Ticks were pooled together by species, life stage, location, and gender. These pools were then separated into groups of up to five adult ticks per pool for testing purposes. Larval and nymphal ticks were used whole (up to 20 specimens per pool) for testing which resulted in 97 total tick pools. The QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) was utilized by manufacturer's recommendations to extract DNA from tick pools in each species.

DNA BARCODING (BARCODE OF LIFE DATA SYSTEM)

Tick species were identified using a dicotomous key, however, coordination with the Walter Reed Biosystematics Unit (WRBU) can produce barcoded species verification using PCR. With time restrictions for this thesis work, coordination could not be completed, however, before scientific publication of this research, verification is advantageous and will be complete. Extracted tick DNA can be barcoded with WRBU's PCR technique with DNA from identified species and cross-referenced to the same species DNA to confirm identification. Information will be entered to the the Barcode of Life Data Systems (BOLD) to provide a platform for collaboration across geographically distinct research communities [15]. By verifying proper identification of species, researchers have the ability to rule out possible cryptic species that may alter the understanding of disease reservoirs, migration and manifestation in Belize as well as expose species divergence [54].

QPCR TESTING OF DNA SAMPLES

The most widely used technique for tick identification and detection of vector-borne pathogens is through the PCR base methodology of real-time quantitative PCR (qPCR) and DNA sequencing [25,37,44]. This study used qPCR methods from previously extracted tick DNA to detect *B. burgdorferi* and *C. burnetii*. All plasmid and primers used in this study were obtained from the Viral and Rickettsial Diseases Department, Infectious Diseases Directorate of the Naval Medical Research Center (NMRC). In order to obtain the most accurate data, each sample was duplicated in a 96 well plate for each reaction. A master mix was prepared from all the reaction components, except sample, under a sterile hood. By using this method of creating the master mix steriley, chances of cross-well contamination and other pipetting errors were reduced.

***Borrelia burgdorferi* qPCR**

The plasmid used to screen samples for *B. burgdorferi* was derived from the 23s rRNA gene in the bacteria and it was synthesized and cloned at NMRC into a pUC19 vector and, therefore, rightfully named by NMRC as BB-23s-pUC19. This closed circular plasmid was purified using the standard Qiagen plasmid mini kit (Qiagen, Stockach, Germany) following the manufacturer's instruction. The pure BB-23s-pUC19 was quantified using a Nano-drop 2000 microsample spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and used as a standard. According to NMRC, the plasmid itself is Ampicillin resistance. The size of the plasmid is 2860 bp with insert of 160 bp. The DNA concentration of this plasmid was 41.7 ng/ μ l and with the plasmid size of 2860 bp,

this results in 1.35×10^{10} copies per μl (calculated from [URI Genomics & Sequencing Center](#)). Subsequent serial dilutions for this plasmid for use as the standard in qPCR are shown in Table 3.

Table 3: <i>Borrelia burgdorferi</i> dilutions for qPCR							
Dilution #	Source of plasmid DNA for dilution	Initial concentration (cp/ μl) C_1	Volume of plasmid DNA (μl) V_1	Volume of diluent (μl) V_2	Final Volume (μl)	Final concentration of dilution (cp/ μl) C_2	Resulting copy # in reaction
1	stock	13500000000	1	49	50	270000000	n/a
2	Dilution 1	270000000	1	49	50	5400000	n/a
*3	Dilution 2	5400000	4.63	45.37	50	500000	10^6
*4	Dilution 3	500000	5	45	50	50000	10^5
*5	Dilution 4	50000	5	45	50	5000	10^4
*6	Dilution 5	5000	5	45	50	500	10^3
*7	Dilution 6	500	5	45	50	50	10^2
*8	Dilution 7	50	5	45	50	5	10^1

*used for standard curve

Primary screening for *B. burgdorferi* was conducted with 18 μl of master mix and 2 μl of DNA template, however, due to discrepancies, this was later changed to 15 μl of master mix and 5 μl of DNA template for testing in each well with a total of 20 μl for each reaction. Master mix was comprised of 10 μl of 2x SYBR® Green (fluorescent DNA-binding dye), 0.14 μl each of 100 μM forward and reverse primer and 4.72 μl of nuclease-free water per reaction per well [11]. Each reaction contained six replicated plasmid samples (standard curve) as positive controls and sterile water as the negative control.

The ABI 7500 FAST real time PCR instrument and program was used for analysis. An initial 10 minute activation step at 95°C was followed by 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds. A standard curve analysis was used to interpret the data. The standard curve for the *B. burgdorferi* reaction is shown in Figure 3. All positive outcomes from qPCR were repeated for positive verification.

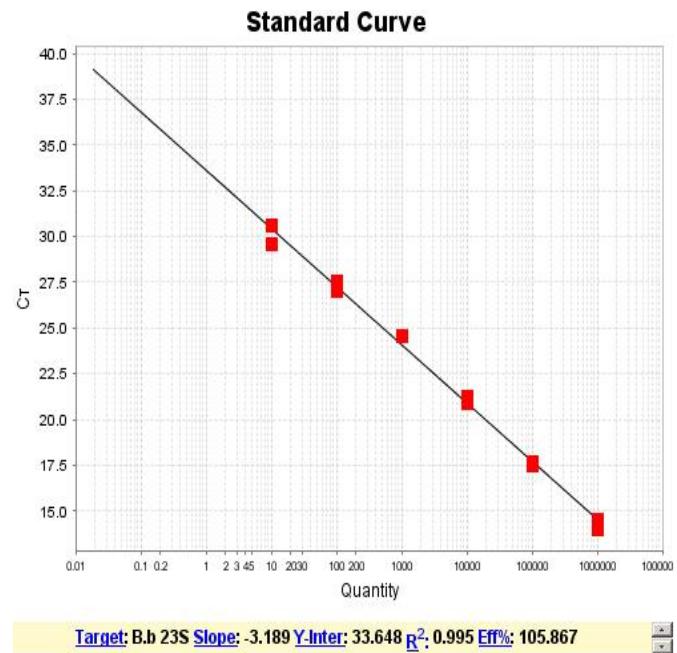


Figure 3: *B. burgdorferi* Standard Curve for qPCR

Coxiella burnetii qPCR

The gene IS1111a of *C. burnetii* RSA 493 was cloned into a pET24a vector. The closed circular plasmid (pET24a-IS1111a) was purified using standard Qiagen plasmid mini kit (Qiagen, Stockach, Germany) following the manufacturer's instruction. The pure pET24a-IS1111a was quantified using a Nano-drop 2000 microsample spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and used as a standard. The DNA concentration of this plasmid was 50.7 ng/ μ l and with the plasmid size of 6300 bp, this results in 7.46×10^9 copies per μ l (calculated from [URI Genomics & Sequencing Center](#)). Subsequent serial dilutions of this plasmid for use as the standard in qPCR are shown in Table 4.

Table 4: Coxiella burnetii dilutions for qPCR

Dilution #	Source of plasmid DNA for dilution	Initial concentration (cp/ μ l) C_1	Volume of plasmid DNA (μ l) V_1	Volume of diluent (μ l)	Final Volume (μ l) V_2	Final concentration of dilution (cp/ μ l) C_2	Resulting copy # in reaction
1	stock	74600000000	1	49	50	149200000	n/a
2	Dilution 1	149200000	1	49	50	2984000	n/a
3	Dilution 2	2984000	16.76	33.24	50	1000000	n/a
*4	Dilution 3	1000000	10	40	50	200000	10^6
*5	Dilution 4	100000	5	45	50	20000	10^5
*6	Dilution 5	10000	5	45	50	2000	10^4
*7	Dilution 6	1000	5	45	50	200	10^3
*8	Dilution 7	100	5	45	50	20	10^2
*9	Dilution 8	10	5	45	50	2	10^1

*used for standard curve

In order to conduct screening for *C. burnetii*, 15 μ l of master mix and 5 μ l of DNA template were added to each testing well for a total of 20 μ l. Master mix for each well was comprised of 10 μ l of 2x SYBR® Green (fluorescent DNA-binding dye), 0.05 μ l each of 200 μ M forward and reverse primer per reaction and 4.9 μ l of nuclease-free water. Each reaction will also have replicated six plasmid samples (standard curve) as positive controls and sterile water as the negative control.

The ABI 7500 FAST real time PCR instrument and program was used for analysis. An initial 10 minute activation step at 95°C was followed by 40 cycles of

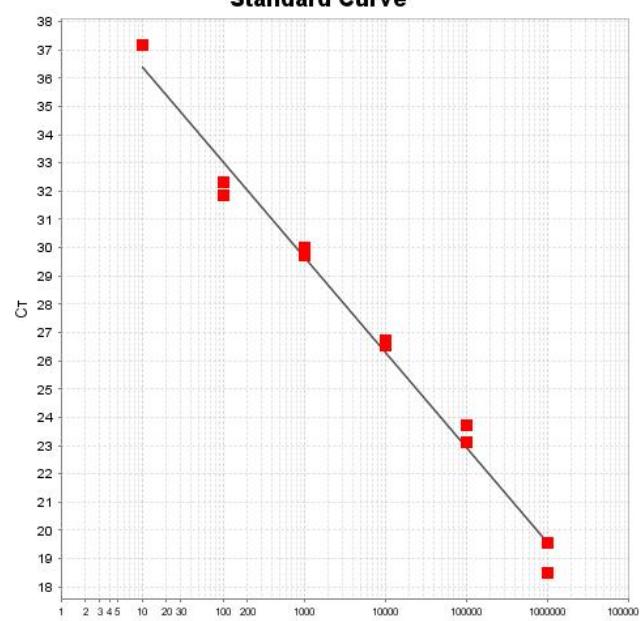


Figure 4: *C. burnetii* Standard Curve for qPCR

95°C for 15 seconds and 60°C for 30 seconds [9]. A standard curve analysis was used to

interpret the data. The standard curve for my *C. burnetii* reaction is shown in Figure 4.

All positive outcomes from qPCR were repeated for positive verification.

Whole Evidence Approach

Some molecular assays may concentrate species pooling and pathogen testing based on known vector competencies and capacities. In other words, tick pathogen screening in the United States may only focus on *Ixodes scapularis* for *B. burgdorferi*, since this is the vector of most concern in the region. By adopting the whole evidence approach of screening all collected tick species and life stages from Belize for *B. burgdorferi* and *C. burnetii*, the knowledge gap recedes for the vector capacity of the region. With this research being the first to test these particular tick-borne pathogens in Belize, screening every specimen collected is ideal.

DATA ANALYSIS

For each climatic season (wet and dry) the following data were analyzed: percentage of infected tick pools and percentage of specimens collected. Risk difference between the quantities of pooled specimens collected in the different climatic seasons and infected tick pools were evaluated using Fisher's exact test, considering p<0.05 as statistically significant.

CHAPTER 3: Results

qPCR RESULTS

All 97 tick pools collected were tested for the presence of *C. burnetii* (Q fever) and *B. burgdorferi* (Lyme disease) using SYBR Green-based qPCR assays as described previously. Of these, zero (0%) ticks tested positive for the presence of *C. burnetii* DNA, of which one possible positive specimen could be identified. Three (3%) tick pools tested positive for the presence of *B. burgdorferi* DNA, one positive pool containing five male *Amblyomma maculatum* ticks and two tick pools containing 20 each of *Dermacentor nitens* larvae.

***Borrelia burgdorferi* qPCR Results**

Three reactions (Figures 5-7) were completed to detect *B. burgdorferi* in all 97 tick pools with confirmation screening. The November samples are shown in the Figure 5. Note around cycles 36-38, a majority of samples seemed to show a positive result. With this indication, the determination was made to use 5 µl of DNA template instead of the original 2 µl of DNA template for better results. Figure 6 shows the February samples and Figure 7 is the overflow from February and any possible positive samples. Possible positive samples were analyzed by melting curve comparisons for validation. Three tick pools (one *A. maculatum* and two pools of *D. nitens* larvae) were positive for *B. burgdorferi*.

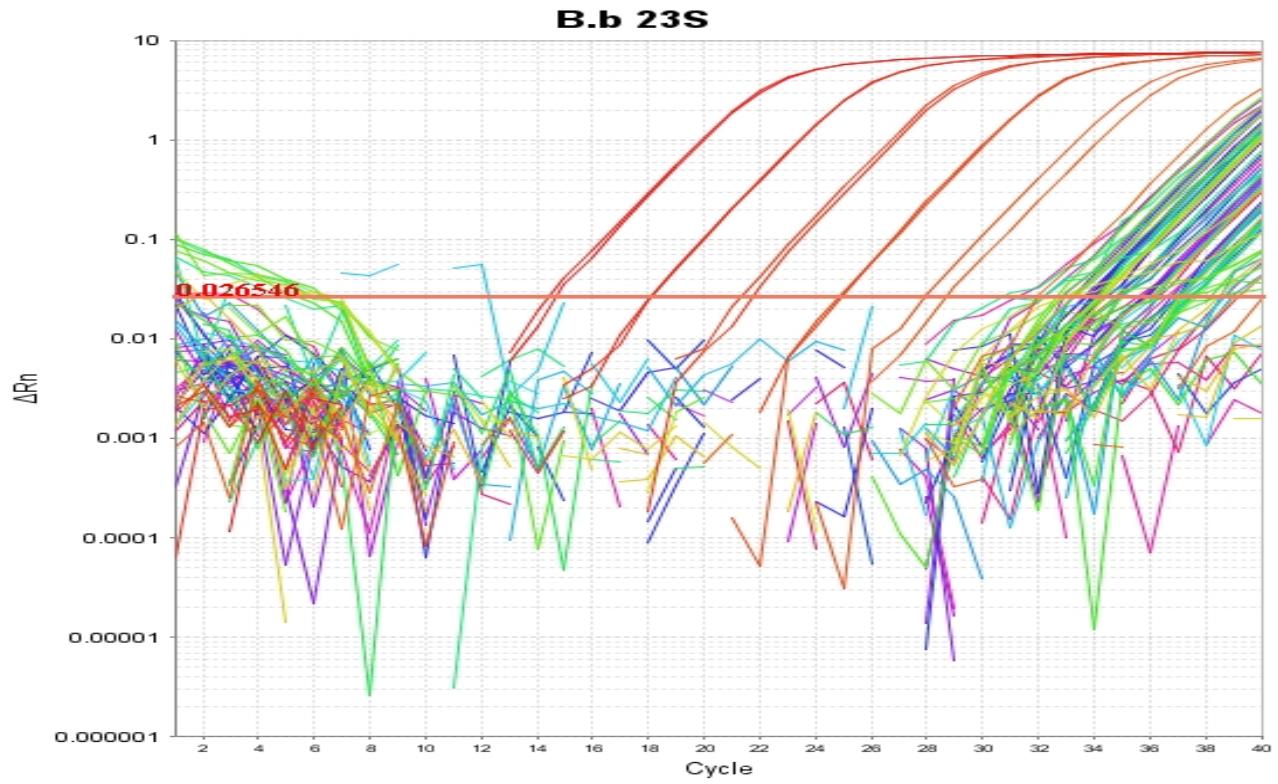


Figure 5: November *B. burgdorferi* qPCR

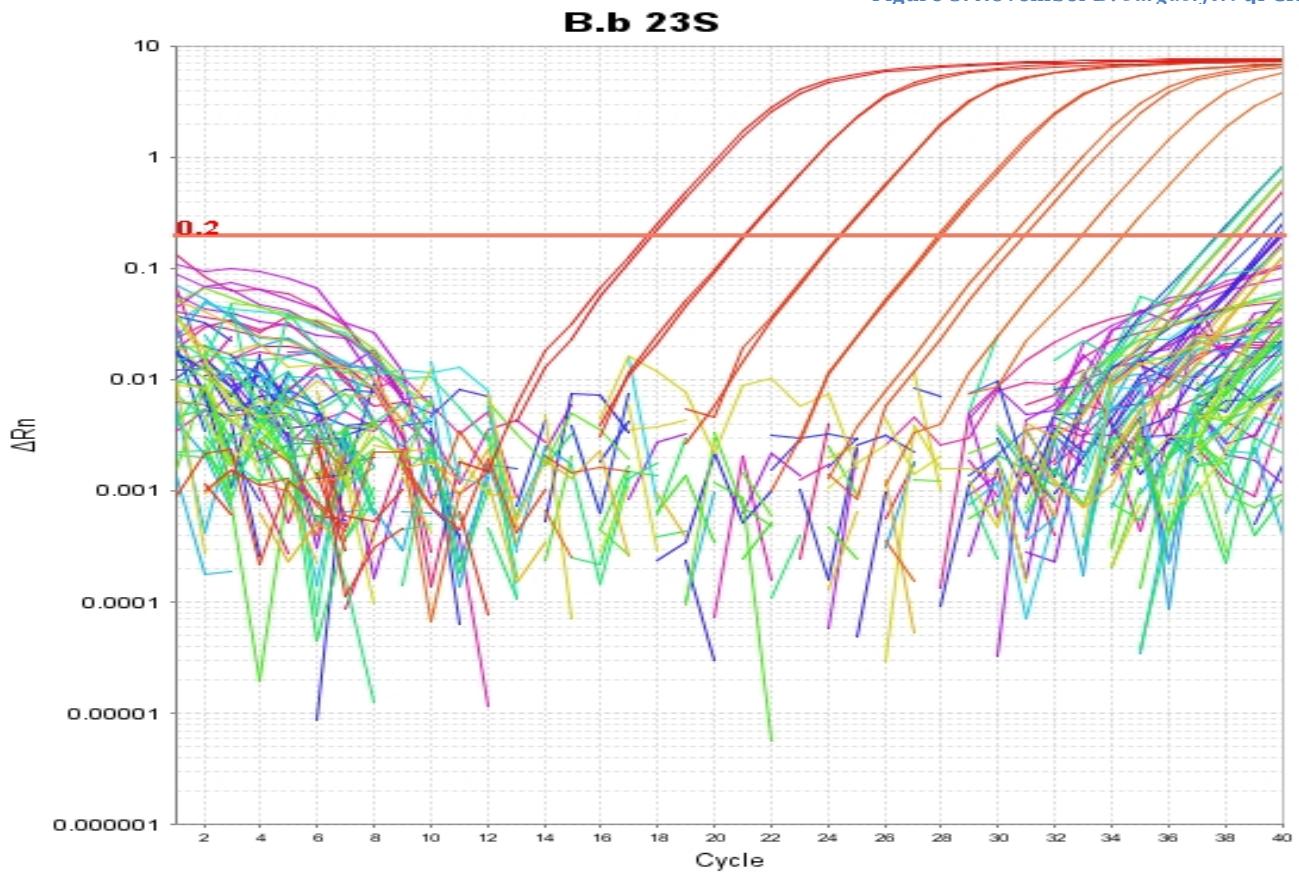


Figure 6: February *B. burgdorferi* qPCR

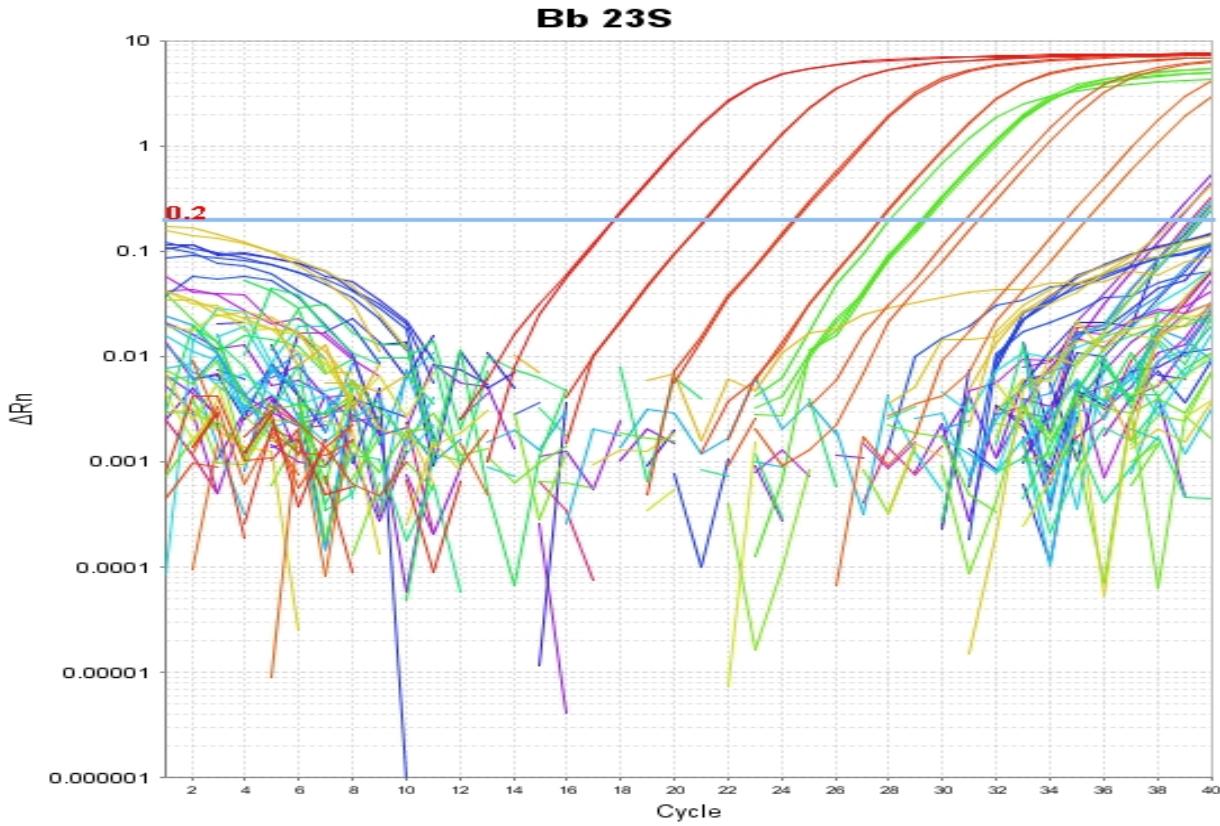


Figure 5: *B. burgdorferi* Feb Overflow & Confirmation qPCR

***Coxiella burnetii* qPCR Results**

Three reactions (Figures 8-10) were completed to detect *C. burnetii* in all 97 tick pools. During primary screening of the February specimens, one tick pool containing one female *Ixodes affinis* (collected from the body of a dog) appeared positive (blue line in Figure 8). Little is known of whether *I. affinis* is a capable human vector for *B. burgdorferi*. It has been found that some Coxiella-like bacteria, belonging to a clade containing *Ornithodoros rostratus*, *O. peruvianus*, and *O. capensis* and a clade containing *Rhipicephalus annulatus*, *R. decoloratus*, *R. geigyi*, *O. sonrai*, and *O. occidentalis*, yield positive PCR results when screened with primers initially believed to be *C. burnetii*-specific [46]. This includes the IS1111 primers used in this study. Further testing using a different target gene may verify whether or not the *I. affinis* sample is a false positive.

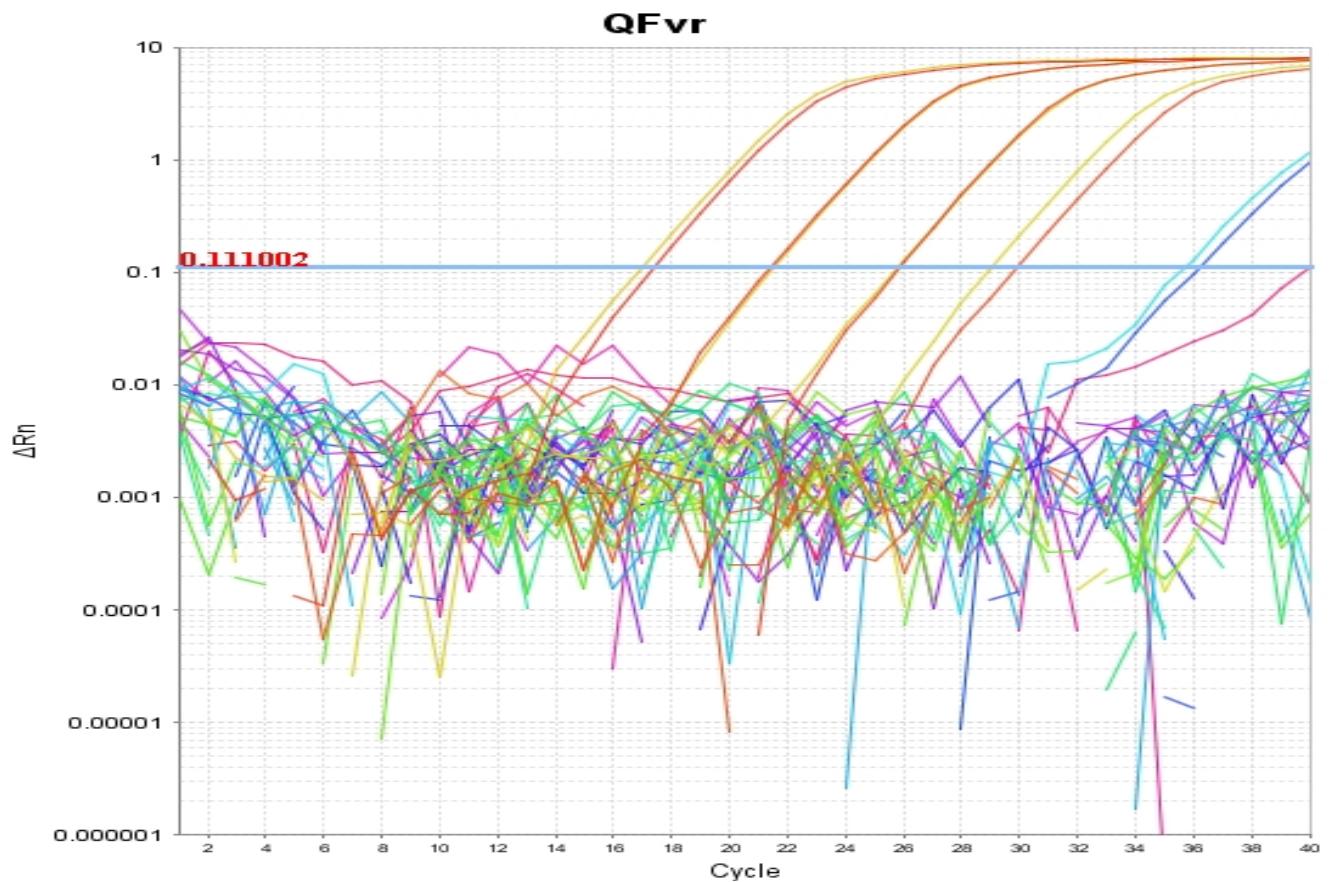


Figure 8: *C. burnetii* February qPCR

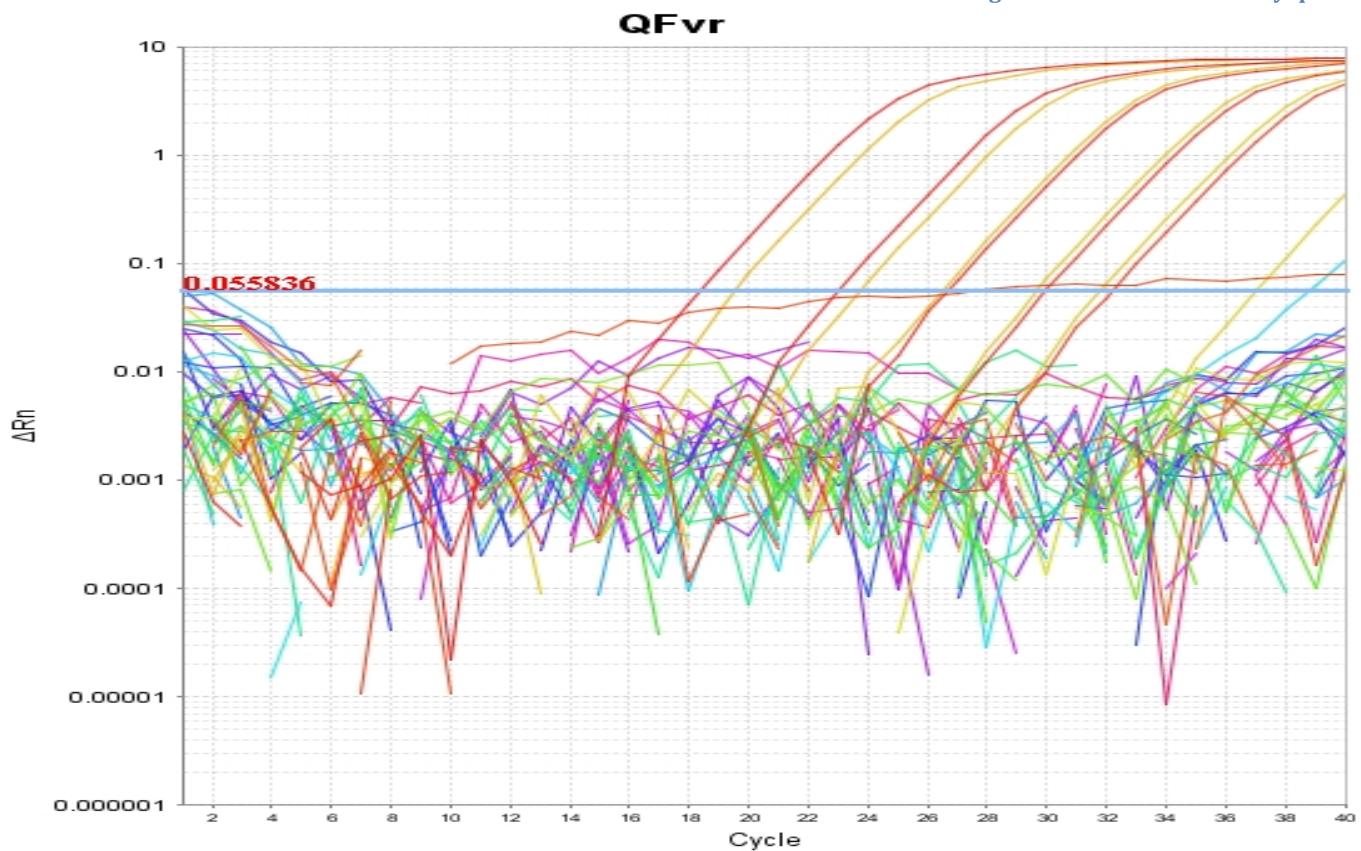


Figure 9: *C. burnetii* November qPCR

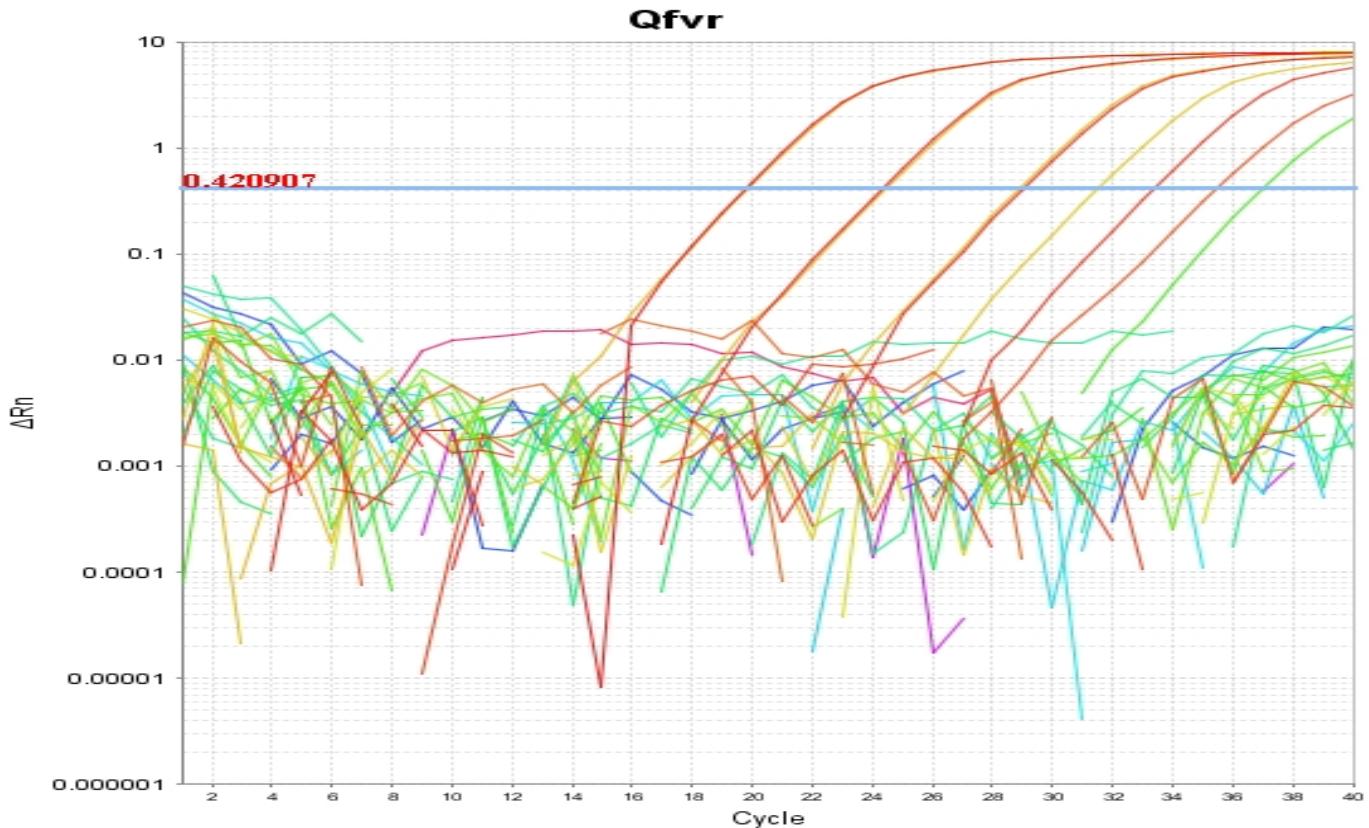


Figure 6: *C. burnetii* February Overflow & Confirmation qPCR

STATISTICAL ANALYSIS

The risk difference was calculated between wet and dry seasons of infected ticks using the contingency table below (Table 5) for both pathogens tested. Data analysis was conducted using Fisher's exact test. A p-value of 0.05 based on two-sided tests was used as the cut-off point to determine statistical significance in all analyses. There were no cases of *C. burnetii* positively identified from the tick pools in this study, but prevalence for *B. burgdorferi* was analyzed. Three of the 97 total tick pools (3%) were positive for *B. burgdorferi*. No co-infections incurred among the 97 ticks pools sampled. All statistical analyses were conducted using IBM SPSS Statistics for Macintosh, Version 22.0 (IBM Corp, 2013).

Table 5: Seasonal Contingency Table			
Season	<i>B.b/C.b(+)</i>	<i>B.b /C.b(-)</i>	Total
Dry Season (Feb)	3	54	57
Wet Season (Nov)	0	40	40
Total	3	94	97

Seasonal Risk Difference

A risk difference can be calculated even when no (zero as denominator) events occur in a group, like no detection of *C. burnetii*. Instead of comparing two measures of disease frequency by calculating their ratio, one can compare them in terms of risk (or absolute) difference. Since ticks were pooled together for testing purposes, exact number of infected ticks could not be detected. This pooling method lowered the sample size and therefore, Fisher's exact test was used to determine the association between seasonal climatic conditions and presence of *B. burgdorferi* /*C. burnetii*. The two-tailed p value = 0.2655 was derived from this analysis. The association between climatic conditions and pathogen presence is considered to be not statistically significant. Ultimately, more sampling and surveillance should be conducted in future research in order to obtain a greater sample size.

Prevalence of *Borrelia burgdorferi*

Dermacentor nitens was found to be the predominant tick species (42.3%) in the area (Table 2), however, the majority collected were larva from a tick drag. From a total of 115 *D. nitens* ticks (19 tick pools) collected in central Belize, *B. burgdorferi* was detected in 2% of the total tick pools examined. Most research for Lyme borrelia in

Central America focuses on *Ixodes* and *Amblyomma* tick vectors. In this study, *B. burgdorferi* was found in one of the 27 (3.7%) *Amblyomma maculatum* tick pools tested. Table 4 outlines the prevalence of *B. burgdorferi* in tested tick pools. For pools resulting in zero infected ticks, the upper tail confidence interval was calculated by the modified Wald method.

Table 6- <i>Borrelia burgdorferi</i> prevalence			
Tick Species	# pools screened	# pools positive	Prevalence (95% CI)
<i>Amblyomma mixtum</i>	33	0	0 (.1239 ^a)
<i>Amblyomma maculatum</i>	27	1	3.7 (-.0342-.10827)
<i>Amblyomma oblongoguttatum</i>	2	0	0 (.7098 ^a)
<i>Amblyomma ovale</i>	5	0	0 (.4891 ^a)
<i>Dermacentor nitens</i>	19	2	10.5 (-.0327-.24326)
<i>Ixodes affinis</i>	2	0	0 (.7098 ^a)
<i>Rhipicephalus microplus</i>	6	0	0 (.4428 ^a)
<i>Rhipicephalus sanguineus</i>	6	0	0 (.4428 ^a)

^aupper tail computed with 95% CI by modified Wald method

Co-infection of *Borrelia burgdorferi* and *Coxiella burnetii*

Although no co-infection of *B. burgdorferi* and *C. burnetii* was detected in this study (table 5), further research and surveillance is needed. Ticks often co-feed on hosts and have been known to share multiple pathogens this way. Co-feeding infections have been detection between *B. burgdorferi* and *C. burnetii* in avian hosts in central Italy [52]. In the same manner, humans that become infected from a tick bite may be exposed to multiple pathogens. Co-infections have been found to occur in human patients in Europe with *C. burnetii*; however, acute Q fever is very often asymptomatic [47]. Precautions should be taken to avoid tick bites in general, but if exposed to tick-borne pathogens, routine testing for concurrent infections with *C. burnetii* is advised.

Table 7- Detection of *Borrelia burgdorferi* and *Coxiella burnetii* in field-collected ticks

Species	# of pools tested	Detection of <i>B.b</i> (%)	Detection of <i>C.b</i> (%)	Co-infection of <i>B.b</i> and <i>C.b</i> (%)
<i>A. mixtum</i>	33			
Male	12	0 (0)	0 (0)	0 (0)
Female	11	0 (0)	0 (0)	0 (0)
Nymph	10	0 (0)	0 (0)	0 (0)
Larva	0	0 (0)	0 (0)	0 (0)
<i>A. maculatum</i>	27			
Male	17	1 (5.9)	0 (0)	0 (0)
Female	9	0 (0)	0 (0)	0 (0)
Nymph	1	0 (0)	0 (0)	0 (0)
Larva	0	0 (0)	0 (0)	0 (0)
<i>A. oblongoguttatum</i>	2			
Male	1	0 (0)	0 (0)	0 (0)
Female	1	0 (0)	0 (0)	0 (0)
Nymph	0	0 (0)	0 (0)	0 (0)
Larva	0	0 (0)	0 (0)	0 (0)
<i>A. ovale</i>	5			
Male	1	0 (0)	0 (0)	0 (0)
Female	4	0 (0)	0 (0)	0 (0)
Nymph	0	0 (0)	0 (0)	0 (0)
Larva	0	0 (0)	0 (0)	0 (0)
<i>D. nitens</i>	19			
Male	4	0 (0)	0 (0)	0 (0)
Female	9	0 (0)	0 (0)	0 (0)
Nymph	3	0 (0)	0 (0)	0 (0)
Larva	3	2 (67)	0 (0)	0 (0)
<i>I. affinis</i>	2			
Male	0	0 (0)	0 (0)	0 (0)
Female	2	0 (0)	0 (0)	0 (0)
Nymph	0	0 (0)	0 (0)	0 (0)
Larva	0	0 (0)	0 (0)	0 (0)
<i>R. microplus</i>	3			
Male	1	0 (0)	0 (0)	0 (0)
Female	1	0 (0)	0 (0)	0 (0)
Nymph	1	0 (0)	0 (0)	0 (0)
Larva	0	0 (0)	0 (0)	0 (0)
<i>R. sanguineus</i>	6			
Male	1	0 (0)	0 (0)	0 (0)
Female	4	0 (0)	0 (0)	0 (0)
Nymph	1	0 (0)	0 (0)	0 (0)
Larva	0	0 (0)	0 (0)	0 (0)
Total	97	3 (3.1)	0 (0)	0 (0)

CHAPTER 4: Discussion

This study is the first to document tick-borne *B. burgdorferi* in Belize and also the first to test for tick-borne *C. burnetii* in this region of Central America. Increasing numbers of Lyme disease cases from humans and animals are emerging from Central and South America. Isolated cases have been reported in some areas of the Caribbean Islands, Argentina, Brazil, Bolivia, Colombia, Costa Rica, Cuba, Chile, Panama, and Mexico. PCR analysis of previously field-collected tick DNA determined the presence of *B. burgdorferi* and revealed the need for modified research by using a different target gene when testing for *C. burnetii*. No occurrence of *B. burgdorferi* and *C. burnetii* co-infections were found in this study. Fisher's exact test determined no significant association between pathogen presence and climatic conditions exist. Future use of molecular techniques (DNA barcoding), to verify vector genus and species of positive results is ideal before scientific publication of results from this study.

IMPORTANCE OF SPECIES

Dermacentor nitens

With focus on *B. burgdorferi*, the primary infected species were *Dermacentor nitens*. This genus can be found worldwide (minus Australia) and includes about 33 species. As stated earlier, the first record of *B. burgdorferi* in *Dermacentor nitens* was isolated from Brazil in 2008 from ticks collected from horses [7]. Although horses have been known as the primary food source for *D. nitens*, they have been found feeding on humans [20]. Detecting *Dermacentor nitens* is capable of carrying *B. burgdorferi* does not necessarily mean vector capacity is detected. Further research is needed on whether

D. nitens will choose a human host over their preferred equine hosts. Another indicator is vector capacity of *D. nitens* and depends on how the population numbers fluctuate by season in Central America. Two positive *B. burgdorferi* pools from this study included 20 larval *D. nitens* specimens per pool. Some research focuses only on engorged ticks and may be missing the opportunity to identify this pathogen in immature ticks.

Amblyomma maculatum

One positive tick pool from this study included five male *Amblyomma maculatum* ticks. This is consistent with the two previously mentioned *A. maculatum* ticks found with *B. burgdorferi* in an Arkansas study in 2012 [18]. Although this tick also predominantly prefers livestock hosts, *A. maculatum* has been found infected with rickettsial organisms of unknown pathogenicity for humans in Peru [5].

CHAPTER 5: Conclusion

SUMMARY OF KEY FINDINGS

This study provides first-time records of tick-borne *B. burgdorferi* found in field-collected ticks from central Belize, Central America. Eight separate tick species were collected from central Belize, showing the diversity in this small geographical area of Belize. Although DNA barcoding was not completed for this study, the Barcode of Life Dataset (BOLD) was cross-referenced for Central American tick species. Seven of the eight tick species for this study were previously entered in BOLD. However, *Amblyomma mixtum* is currently missing from the BOLD dataset. This may be due to its recent reclassification. Entering this molecular data may provide a new platform for research of the history of this species in Central America.

KEY RECOMMENDATIONS

Before scientific publication of this paper, DNA barcoding is necessary to rule out cryptic species and confirm positive identification of species. As stated in the results section, rescreening for *C. burnetii* with a more specific primer set will rule out possible false positives from this study. Also, with recent publications on the spread of other borreliosis like *Borrelia miyamotoi* and *B. mayonii*, it would be interesting to discover if these pathogens have also migrated or previously existed in Central America. This study hopes to open doors to exploring whether other tick-borne pathogens that may be spreading in areas that have thus far not been investigated in Belize.

Disclaimer

Training in appropriate laboratory best practices and chemical safety was given in advance of lab work. Pathogen testing was carried out at the USUHS entomology lab, under the guidance of Dr. Chien-Chung Chao (NMRC) and assistance of Dr. Hua-Wei Chen. All safety measures were strictly adhered to, according to the biosafety level (BSL) of the pathogens tested. Coordination for systematic studies are proposed to the Smithsonian's Museum Support Center, Entomology Division, Walter Reed Biosystematics Unit, in Silver Spring, Maryland, under the guidance of Dr. Yvonne-Marie Linton, BSc (Hons.), PhD, Research Entomologist, WRBU, and Adjunct Assistant Professor, PMB, USUHS, Bethesda, Maryland.

USUHS PhD candidate student, Suppaluck Polsomboon, carried out tick collections in suitable habitats across Belize.

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